

Exhibit 1

## Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice

(tet system/genetic switch/kinetics of induction/liver-specific control)

ANDREAS KISTNER<sup>†</sup>, MANFRED GOSSEN<sup>†‡</sup>, FRANK ZIMMERMANN<sup>†</sup>, JASNA JERECIC<sup>†</sup>, CHRISTOPH ULLMER<sup>§</sup>, HERMANN LÜBBERT<sup>§</sup>, AND HERMANN BUJARD<sup>†¶</sup>

<sup>†</sup>Zentrum für Molekulare Biologie der Universität Heidelberg, ZMBH, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany; and <sup>§</sup>Sandoz Pharma, Ltd., CH-4002 Basel, Switzerland

Communicated by Klaus Rajewsky, Universität Köln, Köln, Germany, July 8, 1996 (received for review April 25, 1996)

**ABSTRACT** The tet regulatory system in which doxycycline (dox) acts as an inducer of specifically engineered RNA polymerase II promoters was transferred into transgenic mice. Tight control and a broad range of regulation spanning up to five orders of magnitude were monitored dependent on the dox concentration in the water supply of the animals. Administration of dox rapidly induces the synthesis of the indicator enzyme luciferase whose activity rises over several orders of magnitude within the first 4 h in some organs. Induction is complete after 24 h in most organs analyzed. A comparable regulatory potential was revealed with the tet regulatory system where dox prevents transcription activation. Directing the synthesis of the tetracycline-controlled transactivator (tTA) to the liver led to highly specific regulation in hepatocytes where, in presence of dox, less than one molecule of luciferase was detected per cell. By contrast, a more than 10<sup>5</sup>-fold activation of the luciferase gene was observed in the absence of the antibiotic. This regulation was homogeneous throughout but stringently restricted to hepatocytes. These results demonstrate that both tetracycline-controlled transcriptional activation systems provide genetic switches that permit the quantitative control of gene activities in transgenic mice in a tissue-specific manner and, thus, suggest possibilities for the generation of a novel type of conditional mutants.

Most insights into gene function have been gained from the study of organisms susceptible to both efficient genetic dissections and biochemical analysis. Higher eukaryotes and particularly mammals do not belong to this category. The complexity of the genome, embryonic development, long generation times, and the difficulty of studying large numbers of individuals make the genetic analysis of these systems difficult, if not impossible. The technique of gene targeting in mice (1) has been an important breakthrough, but the irreversibility of the mutational alterations, which may lead to compensatory developments, developmental defects, and even embryonic mortality, limit this approach. One way to partially overcome such limitations is gene targeting with the site-specific Cre/lox recombination system (2) as pioneered by Byrne and Ruddle (3), by Westphal and coworkers (4), and particularly by K. Rajewsky and coworkers (5, 6). In this strategy, the CRE recombinase, controlled by an appropriate promoter, is used to activate, inactivate, or alter a gene during a defined differentiated state of cells in the developing organism. Again, however, the genetic changes are irreversible and follow a program that cannot be influenced after its onset.

A "genetic switch" that could be operated at will and that would permit the control of individual gene activities quantitatively and reversibly in a temporal and spatial manner would

thus be of great advantage. The tetracycline (Tc)-controlled systems for the activation of transcription (7, 8) fulfill a number of these requirements at the cellular level. Herein, we report that the "reverse Tc-controlled transactivator" (rtTA) system, where doxycycline (dox) acts as an inducer of transcription as well as the "Tc-controlled transactivator" (tTA) system, where Tc or dox prevent transcription activation (Fig. 1) can be operated in a quantitative and highly tissue-specific way when transferred into mice. The results show that the controls are tight and that the kinetics of induction, especially with the rtTA system, are rapid. Although we (9, 10) and others (11, 12) have reported that the tTA system can be applied to transgenic organisms, the results reported herein establish that both the rtTA and the tTA systems provide true genetic switches capable of quantitatively controlling individual gene activities in animals in a highly tissue-specific manner. These observations open up exciting prospects for the study of gene function in mammalian organisms.

### MATERIALS AND METHODS

**Plasmid Constructs.** Plasmid pUHG17-1 is derived from pUHD17-1 (8) by replacing the simian virus 40 poly(A) signal with the  $\beta$ -globin intron poly(A) signal. The liver-specific tTA expression plasmid pUHG15-30 contains the tTA gene under the control of P<sub>LAP</sub>, the promoter of the liver-enriched activator protein. It was obtained by cloning a 2.9-kb *EcoRI*-*ApaI* fragment containing the LAP promoter (13) in front of the tTA gene of pUHG15-1 (10). For details, see <http://ix.urz.uni-heidelberg.de/~q35/>.

**Transgenic Animals.** Transgenic mouse lines (NMRI outbred) were generated by pronuclear injection using standard techniques (14) and analyzed by the Southern blot technique (15) using the *Bam*HI-*Eco*RV fragment of the luciferase gene and the *Xba*I fragment of the tTA gene as respective probes. Luciferase reporter mice were obtained upon transfer of the 3.1-kb *Xho*I-*Eae*I fragment of pUHC13-3 (7). Mice producing rtTA controlled by P<sub>hCMV</sub>, the promoter of the human cytomegalovirus immediate early genes, were obtained upon transfer of the 2.7-kb *Pf*IMI-*Xho*I fragment and animals synthesizing tTA under the control of the LAP promoter were obtained by transferring the 5.5-kb *Ase*I-*Asp*700 fragment of pUHG15-30. Animals transgenic for both a transactivator and a reporter unit were exposed when necessary to doxycycline hydrochloride (Sigma) dissolved in 5% sucrose supplied as drinking water, which was exchanged every 3 days. Possible long-term effects of dox (200  $\mu$ g/ml) were examined in a 3-month trial.

Abbreviations: Tc, tetracycline; rtTA, reverse Tc-controlled transactivator; tTA, Tc-controlled transactivator; dox, doxycycline; rlu, relative light unit(s).

<sup>‡</sup>Present address: Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720-3202.

<sup>¶</sup>To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Exhibit 1

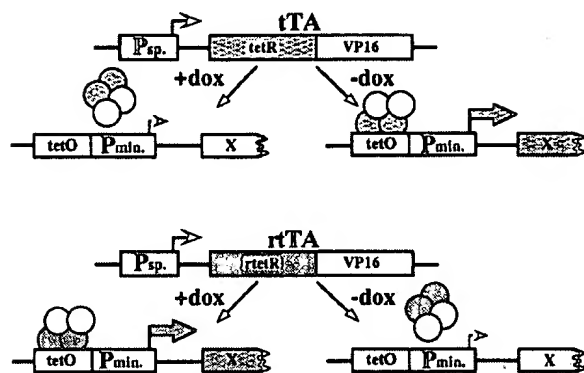


Fig. 1. Schematic outline of the tet regulatory systems (as adapted from ref. 8). (Upper) Mechanism of action of the Tc-controlled transactivator (tTA). The fusion protein is composed of the repressor (tetR) of the Tn10 Tc-resistance operon of *Escherichia coli* and a C-terminal portion of protein 16 of herpes simplex virus that functions as a strong transcription activator. tTA binds in absence of dox but not in its presence to an array of seven cognate operator sequences (tetO) and activates transcription from a minimal human cytomegalovirus (hCMV) promoter, which itself is inactive (7). The minimal promoter-tet operator fusion is referred to as  $P_{hCMV-tet}$ . The lower part shows the rtTA system, which is identical to the tTA system with the exception of 4 amino acid exchanges in the tetR moiety. These changes convey a reverse phenotype to the repressor (rtetR). The resulting rtTA requires dox for binding to tetO and thus for transcription activation (8). Tissue specificity of these systems is achieved by placing the tTA or rtTA gene under the control of a tissue-specific promoter ( $P_{sp}$ ). Thus, in mouse lines where tTA and rtTA synthesis is controlled by  $P_{hCMV}$  ( $TA^{hCMV}$  and  $rTA^{hCMV}$ ), dox-regulated expression of indicator genes is found in a number of tissues whereas the  $P_{LAP}$  led to mouse lines ( $TA^{LAP}$ ) producing tTA in hepatocytes. The indicator mouse line L7 contains the luciferase gene under the control of  $P_{hCMV-tet}$ .

Neither overall toxic effects nor any histological changes in liver and kidney sections were detected (data not shown). Also, no abnormalities in litter size and appearance of the offsprings were observed after pregnancy in the presence of the antibiotic.

**Determination of Luciferase in Mouse Tissue.** Tissue samples rinsed with PBS and homogenized on ice in 500  $\mu$ l of lysis buffer (7) with an Ultraturrax (Janke & Kunkel, Staufen, Germany) were quickly frozen in liquid nitrogen, thawed, and centrifuged for 5 min at  $15,000 \times g$ . Twenty microliters of the supernatants was assayed for luciferase activity as described (7), except that the concentration of D-luciferin (Sigma) was 125  $\mu$ M. Another aliquot was used for protein determination (16). The background signals measured in any tissue of animals that did not carry the luciferase transgene were indistinguishable from the instrumental background [80–150 relative light units (rlu)/10 sec]. Luciferase activity from live animals was determined by using 1–2 mm of tail tissue homogenized in 100  $\mu$ l of lysis buffer. [Note that unlike reports (10, 17), luciferase activities are normalized to  $\mu$ g of protein.] Comparing luciferase in extracts of X1 cells (7) with standard preparations of commercially available enzyme (Boehringer Mannheim) showed that the specific activities are the same. This allowed the calibration of luciferase activity in mouse liver extracts by relating the number of enzyme molecules to the number of hepatocytes per weight of tissue ( $2 \times 10^5$  cells per mg; ref. 18).

**Histology of Liver Specimens.** For identifying tTA and luc mRNA by *in situ* hybridization, oligonucleotide probes (tTA, 5'-CCTAGCTTCTGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCG-3'; luc, 5'-CGTGATGTTCACCTCGATATGTGCATCTGTAAAGCAATTGTTCC-3') were synthesized and 3'-end-labeled with terminal deoxynucleotidyltransferase (Boehringer Mannheim) using a 30:1 molar ratio of deoxyadenosine 5'-[ $\alpha$ - $^{35}$ S]thio]triphosphate (1200 Ci/mmol; 1 Ci = 37 GBq; NEN)

to oligonucleotide (19). Tissue samples were fixed with 4% paraformaldehyde in PBS, perfused with 30% sucrose, and frozen on dry ice. Hybridization and autoradiography were carried out on cryosections mounted onto poly-(L-lysine)-coated slides as described (19). To detect lacZ expression, tissue samples were prepared and 4-bromo-3-chloro-2-indolyl  $\beta$ -D-galactoside staining was performed as described (14).

## RESULTS

**Experimental Strategy.** To delineate the potential of the Tc-controlled activation via rtTA or tTA in transgenic mice, indicator mouse lines carrying the luciferase gene as a highly sensitive indicator function under the control of a rtTA/tTA-responsive promoter were generated as well as animals producing rtTA or tTA under the control of two different promoters. Crossbreeding of rtTA- or tTA-producing animals with "indicator mice" should lead to offsprings where induction factors, induction kinetics, and tissue specificity of expression could be studied. The promoters used to direct the synthesis of rtTA and tTA were  $P_{hCMV}$ , the human cytomegalovirus immediate early promoter (20), and  $P_{LAP}$ , the promoter responsible for the expression of the LAP (C/EBP $\beta$ ) transcription factor in the liver of rats (13). While  $P_{hCMV}$  would direct rtTA or tTA synthesis to a variety of organs,  $P_{LAP}$  was expected to function in the liver where its activity was restricted to parenchymal hepatocytes (U. Schibler and P. Fonjallaz, personal communication).

**Generation of Luciferase Reporter Mouse Lines.** The luciferase gene controlled by  $P_{hCMV-tet}$  (7) was transferred into mice by pronucleus injection. Of 38 potential founder animals, Southern blot analysis revealed 10 (L0 to L9) carrying the transgene. A low luciferase activity was detected in the tail tissue of 6 animals. When cultures of primary ear fibroblast from the 10 founders were transfected with tTA-expressing DNA (pUHD15-1; ref. 7), 7 showed Tc-dependent stimulation of luciferase activity (21) of which 5 transmitted the transgene. Monitoring the luciferase activity in 12 organs of these lines revealed line L7 where the basal luciferase activity was very low and constituted a characteristic and stable pattern (see Fig. 4). The lowest basal activity was found in liver and pancreas ( $\leq 0.03$  rlu/ $\mu$ g of protein). Absolute determination of the luciferase in the liver of L7 animals showed that  $<1$  molecule of the enzyme is present per hepatocyte. The highest background was found in the tongue and exceeded that of the liver about 100-fold when organ extracts were normalized to the protein content.

**Identification of Mouse Lines Producing rtTA or tTA Under the Control of  $P_{hCMV}$ .** Four founders ( $rTA^{hCMV1}$  to  $rTA^{hCMV4}$ ) that carried the transgene  $P_{hCMV-tTA}$ , retrieved from pUHG17-1, were identified among 19 offsprings by Southern blot analysis. Crosses between these lines and L7 mice yielded offsprings carrying both transgenes. They were fed dox (2 mg/ml in the water supply) for 1 week before luciferase activity in various organs was determined. Little or no activation of luciferase was conveyed by the  $rTA^{hCMV1}$  and  $rTA^{hCMV2}$  lines (data not shown). By contrast, high enzyme levels were found in  $rTA^{hCMV3/L7}$  and  $rTA^{hCMV4/L7}$  individuals. Thus, only the  $rTA^{hCMV3}$  and the  $rTA^{hCMV4}$  lines were included in further studies.

Analogous experiments were carried out with four mouse lines producing tTA under the control of  $P_{hCMV}$  ( $TA^{hCMV1}$ ,  $TA^{hCMV2}$ ,  $TA^{hCMV4}$ , and  $TA^{hCMV5}$ ; ref. 10). All offsprings of crosses with the L7 line, which carried both transgenes showed elevated luciferase levels; however, lines  $TA^{hCMV1}$  and  $TA^{hCMV5}$  gave rise to an approximately 100 times higher enzymatic activity than lines  $TA^{hCMV2}$  and  $TA^{hCMV4}$  (data not shown). Only the  $TA^{hCMV1}$  and  $TA^{hCMV5}$  mouse lines were, therefore, studied further.

In all four, the two rTA- and the two tTA-producing mouse lines, the synthesis of the Tc-controlled transactivators was directed by  $P_{hCMV}$ . Consequently, rTA/tTA-dependent regulation was observed primarily in those organs/tissues where this promoter is known to be active—e.g., in muscle, kidney, thymus (22), and pancreas but not in, e.g., liver, lung, and lymphocytes (M.G. and A.K., unpublished results).

**Dox-Dependent Regulation of Luciferase Activity.** The effect of dox on luciferase synthesis in double transgenic mice (i.e., rTACMV3/L7, rTACMV4/L7, and TACMV1/L7) was measured after exposing the animals for 1 week to dox (2 mg/ml) in the drinking water. When the rTACMV3/L7 and rTACMV4/L7 animals were analyzed, two remarkable results were obtained. (i) In absence of dox, luciferase expression was indistinguishable from that of the L7 line in all organs analyzed except for two rTACMV4/L7 animals that showed slightly elevated values in the kidney (Fig. 2A). This demonstrates that the presence of rTA does not cause an intrinsic elevation of the background. (ii) In presence of dox, luciferase synthesis was induced over several orders of magnitude depending on the organ/tissue. Up to a  $10^5$ -fold induction was observed in the pancreas but high luciferase values were also found, e.g., in muscle, stomach, and thymus. Both mouse lines, rTACMV3 and rTACMV4, produced very similar patterns (Fig. 2A) reflecting the activity of  $P_{hCMV}$  in various tissues of transgenic mice. Little or no activity was found in lung, brain, liver, and lymphocytes, never exceeding 7 rlu/ $\mu$ g of protein.

As expected, the opposite effect of dox was observed with TACMV1/L7 animals. In absence of the antibiotic, luciferase was highly induced, again following the tissue pattern of  $P_{hCMV}$  activity in mice (Fig. 2B). Administration of dox reduces the activity of luciferase to background levels in most organs and in pancreas, a regulation factor of almost  $10^5$  was again reached.

**Time Course of Luciferase Induction and Partial Activation.** To study the kinetics of luciferase induction in rTACMV3/L7 mice, animals were switched to dox-containing drinking water and sacrificed after 4, 9, and 24 h for determining luciferase activity in various organs. Significant induction was observed after 4 h in most organs analyzed, and full induction was generally achieved after 24 h (Fig. 3A). When the time course of induction was followed in TACMV1/L7 mice, the animals were kept at the lowest concentration of dox, 20  $\mu$ g/ml, shown to prevent tTA-dependent activation in all organs except skeletal muscle (Fig. 3A). Upon withdrawal of the antibiotic in the water supply, significant induction was found after 24 h in several organs, particularly in tongue, heart, thymus, and pancreas, and almost full activation was monitored after 1 week. As expected, induction of the rTA system was more rapid since it does not rely to the same extent on the biological half-life of dox in the animal.

Partial activation of luciferase synthesis by limiting amounts of dox was achieved by exposing TACMV1/L7 animals to increasing concentrations of dox in the drinking water for 1 week. While tTA action was still prevented with dox at 20  $\mu$ g/ml in all organs except skeletal muscle, concentrations of 2  $\mu$ g/ml and 200 ng/ml led to different levels of activity, however, in an organ-dependent manner. As exemplified in Fig. 3B, with dox at 200 ng/ml, luciferase activity was fully induced in heart but only partially induced in pancreas, whereas little effect was seen in kidney.

**Directing tTA Synthesis to the Liver for Tissue-Specific Regulation.** The transactivator tTA was placed under the control of the cellular promoter  $P_{LAP}$  that carries a cis-acting locus control-like element and can direct transcription to the liver (13). Upon pronucleus injection of the  $P_{LAP}$ -tTA construct, 50 offsprings were obtained of which 5 contained the transgene as evidenced by Southern blot analysis. The 5 founder lines were crossed with L7 mice and the 2 functional lines were designated TAILAP1 and TAILAP2. The double trans-

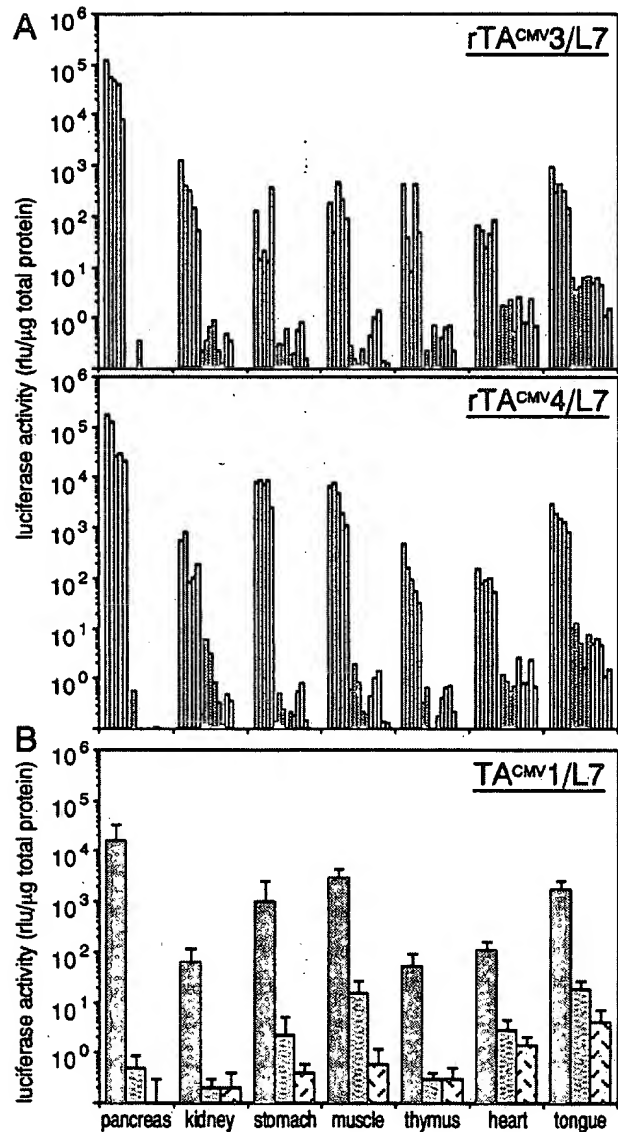


FIG. 2. Luciferase induction in double transgenic rTACMV/L7 and TACMV/L7 mice. (A) Pattern of luciferase activity in various organs of individual rTACMV3/L7 and rTACMV4/L7 animals. Bars: darkly shaded, luciferase activities in absence; lightly shaded, luciferase activity in presence of dox (2 mg/ml, 1 week); open, background activities of L7 animals (note the logarithmic scale). (B) Pattern of luciferase activity in various organs of TACMV1/L7 animals. Bars: darkly shaded, luciferase activities in absence of dox ( $n = 7$ ); lightly shaded, luciferase activities in presence of dox (2 mg/ml, 1 week) ( $n = 5$ ); stippled, average values of the L7 line ( $n = 15$ ). Error bars show standard deviations.

genic animals TAILAP1/L7 and TAILAP2/L7 showed very high levels of luciferase activity in the liver that were reduced to background activity when the animals were exposed to dox at 2 mg/ml in the drinking water (Fig. 4) resulting in a regulation factor of more than  $10^5$  in both lines.

Correlating these enzymatic activities to the number of cells extracted,  $10^4$  to  $10^5$  luciferase molecules were calculated per hepatocyte in the induced state. If, in addition, one accepts that luciferase is about as short lived in these tissues as it is in HeLa cells ( $t_{1/2} \approx 3$  h; ref. 7), it becomes obvious that the fully induced  $P_{hCMV}$  was capable of directing high-level gene expression in animals just as in cell cultures (7, 8, 23).

An exceptionally stringent tissue specificity was observed with the TAILAP2 line: In all organs analyzed, the luciferase

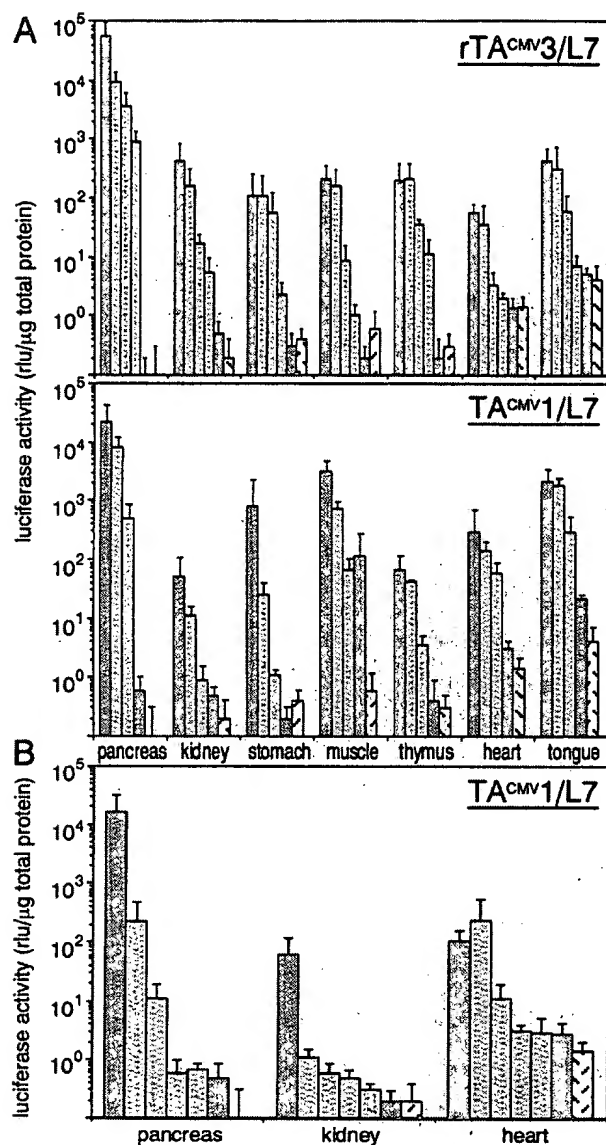


FIG. 3. Kinetics of luciferase induction and partial induction. (A)  $rTACMV3/L7$  mice were exposed to dox at 2 mg/ml in the drinking water and luciferase activity was measured in the defined organs after 4, 9, and 24 h, lightly shaded bars from the right (for each bar,  $n = 4$ ). Darkly shaded bars show steady-state values in absence (right,  $n = 5$ ) and presence (left,  $n = 5$ ) of the antibiotic.  $TACMV1/L7$  animals were exposed to dox at 20  $\mu$ g for 1 week before plain water was administered. Lightly shaded bars show luciferase activity after 24 h (right,  $n = 4$ ) and 1 week (left,  $n = 4$ ). Darkly shaded bars depict luciferase activity in presence (right,  $n = 4$ ) and in absence (steady state) of dox (left,  $n = 9$ ). (B) Partial induction of luciferase in  $TACMV1/L7$  animals. The animals were exposed to different concentrations of dox. The lightly shaded bars depict from right to left luciferase activities in the indicated organs with the antibiotic at 200 ( $n = 3$ ), 20 ( $n = 4$ ), 2 ( $n = 4$ ), and 0.2 ( $n = 3$ )  $\mu$ g/ml in the drinking water (for 1 week). The darkly shaded bars indicate luciferase activities in absence (left,  $n = 7$ ) or presence (right,  $n = 5$ ) of dox (2 mg/ml). In all graphs, the stippled bars depict the L7 background ( $n = 15$ ) and error bars indicate the standard deviations.

activity of  $TA^{LAP2}/L7$  animals was 200- (spleen) to 10,000-fold lower than in the liver whereby in a number of organs this activity was identical to the L7 background (Fig. 4). A relatively high luciferase activity was monitored in the brain of  $TA^{LAP1}/L7$  animals.

*In situ* hybridization of oligonucleotide probes directed towards mRNA of  $tTA$  or luciferase revealed a homogeneous

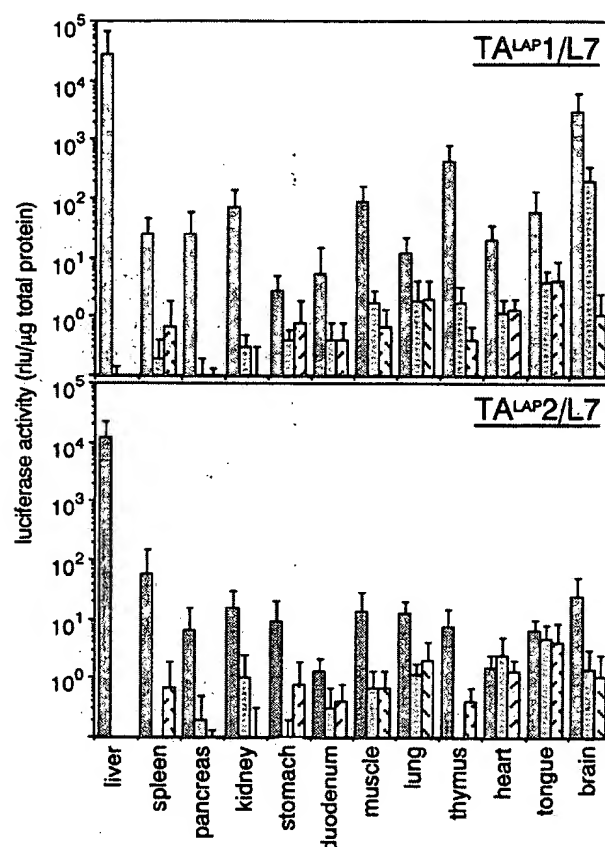


FIG. 4. Liver-specific regulation of luciferase.  $TA^{LAP1}/L7$  and  $TA^{LAP2}/L7$  double transgenic animals were analyzed for luciferase activity in absence (darkly shaded bars,  $n = 4$  or 5) and presence (lightly shaded bars,  $n = 4$  or 5) of dox (2 mg/ml in drinking water). Missing bars indicate that luciferase activity was not measurable. Stippled bars give the L7 background and error bars indicate standard deviations.

staining of livers of  $TA^{LAP1}/L7$  mice in absence of dox (Fig. 5A and B) whereas in its presence, luciferase-specific RNA was not detectable (Fig. 5B). When  $TA^{LAP1}$  mice were crossed with a mouse line containing the  $\beta$ -galactosidase gene under the control of  $P_{hCMV-1}$  (J.J., P. Seeburg, and R. Sprengel, unpublished results), liver sections of animals carrying both transgenes showed a homogeneous staining of hepatocytes in the absence of dox whereby no  $\beta$ -galactosidase activity was detected in cells constituting, e.g., capillary vessels (21).  $\beta$ -Galactosidase activity was eliminated when the animals were kept under dox (Fig. 5C). In sections of other tissues as exemplified for a brain region (Fig. 5D),  $\beta$ -galactosidase was not detectable independent of the absence or presence of dox.

## DISCUSSION

The results reported herein show that the  $rTA$ - as well as the  $tTA$ -based regulatory system permits the control of gene activities in transgenic mice over a wide range and that "off-states" can be achieved that appear equivalent to null mutations. Moreover, induction in many organs is rapid, particularly with the  $rTA$  system. As in HeLa cells (7, 8) and in tobacco plants (9), the tet systems exhibit no measurable "intrinsic leakiness." Instead, the background activities, which are sometimes observed, reflect activation from outside and depend on the chromosomal site where a gene controlled by a  $rTA/tTA$ -responsive promoter is integrated. Interestingly, loci where little or no activation occurs but are nevertheless susceptible to high activation via  $rTA/tTA$  are not too

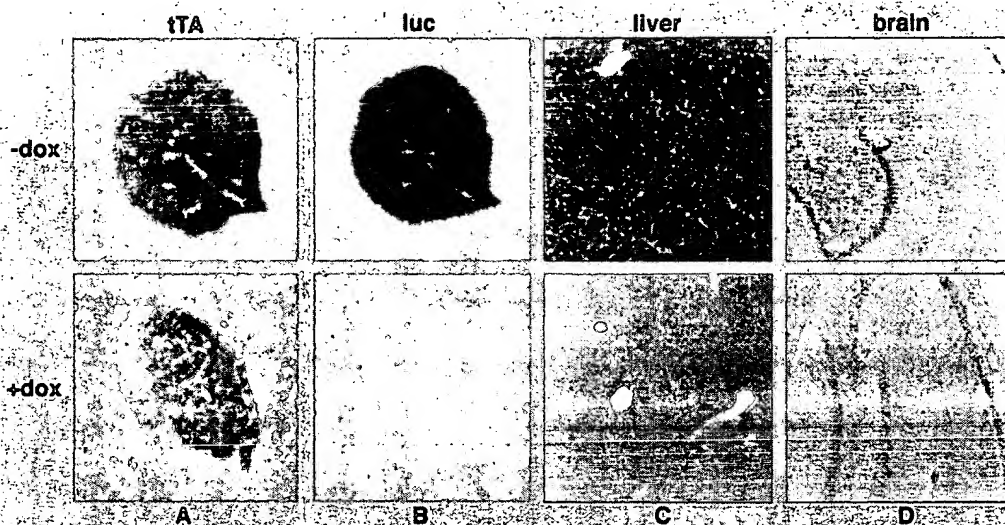


FIG. 5. *In situ* analysis of tTA and luc mRNA in the liver of TA<sup>LAP1</sup>/L7 mice (A and B). Tissue samples of dox-treated (2 mg/ml) and untreated animals were hybridized with radioactively labeled tTA- (A) and luc- (B) specific antisense oligonucleotide probes. Exposure was 15 days for the tTA and 6 days for the luciferase signal. Expression of a tTA-controlled lacZ transgene in liver and brain of TA<sup>LAP1</sup>/lacZ<sup>+</sup> mice (C and D). Liver (C) and brain (D) (hippocampus area) cryosections of animals treated with or without dox (2 mg/ml) were subjected to 4-bromo-3-chloro-2-indolyl  $\beta$ -D-galactoside-staining followed by counterstaining with nuclear fast red.

infrequent. The first examples for such functionally defined loci are the integration sites of the luciferase gene in the X1 and X2 HeLa cell lines (7) that, in presence of dox, produce less than seven molecules of luciferase per cell (M.G., A. Bonin, and A.K., unpublished results) while a more than  $10^5$ -fold induction occurs in the absence of the antibiotic. Another example is provided by the L7 mouse line, where very low luciferase activities are monitored in all organs, particularly in liver, pancreas, and lymphocytes (data not shown). Less than one molecule of the enzyme per cell was calculated by relating the luciferase activity in liver extracts to the number of hepatocytes of the starting material. This indicates that also in other organs where up to 100 times higher luciferase activities were measured (normalized to the protein content of the extracts), extremely low background activity must prevail. This "L7 locus" is activatable in all organs/tissues where rTA or tTA was synthesized: in addition to the 12 organs shown in Fig. 4, the locus functions in various areas of the brain and in lymphocytes (21), suggesting that it is accessible for rTA/tTA in most, if not all cell types.

When the synthesis of rTA is directed by the P<sub>hCMV</sub>, activation of luciferase in presence of dox is observed in a number of tissues/organs (Fig. 2A). The highest activation factors exceeding five orders of magnitude are measured in the pancreas but luciferase activity is increased also in other organs  $10^2$ - to  $10^4$ -fold. An identical pattern of activation is obtained with two P<sub>hCMV</sub>-controlled tTA mouse lines of which one is shown in Fig. 2B. Again, an about  $10^5$ -fold activation is monitored in the pancreas and high activities are measured also in stomach and skeletal muscle. We interpret the different levels of activation in various organs as reflecting the activity of P<sub>hCMV</sub> in different cell types. This is supported by results obtained with other rTA- and tTA-producing mouse lines (TA<sup>CMV2</sup>, TA<sup>CMV4</sup>, and rTA<sup>CMV2</sup>), where the same relative pattern of activity throughout the organs is detected but at 10- to 1000-fold lower levels. The only organ in all animals where dox did not fully reduce tTA activity to background level under the conditions used is skeletal muscle. This deserves further analysis since it contradicts earlier observations (24).

Animals where the transactivator synthesis was specified by P<sub>hCMV</sub> appeared particularly useful for studying kinetics of induction and partial induction. These would allow measurement of kinetics parameters in different organs where the

pharmacokinetic properties of dox might generate different results. As shown in Fig. 3A, rapid induction in rTA<sup>CMV3</sup>/L7 mice is achieved upon supply of dox. In some organs (pancreas, kidney, and thymus), luciferase activity is elevated already after 4 h and in most organs induction is complete after 24 h. These data delineate lower limits of induction kinetics since there are more efficient techniques for administering dox than the oral route. As expected, induction via the tTA system (TA<sup>CMV1</sup>/L7 animals, Fig. 3A) is slower, since herein the effector molecule has to be withdrawn and, consequently, the biological half-life of the antibiotic is a decisive parameter. Thus, while in several organs (pancreas, thymus, heart, and tongue) elevated luciferase levels are seen after 24 h, full induction may take 4–7 days in some organs. In such experiments, it is important to keep the animals initially at the lowest effective dox concentration, which in our experience is 20  $\mu$ g/ml in the water supply (Fig. 3A) (25). Tetracyclines with different biological half-life and compounds acting as Tc antagonists would certainly be useful to accelerate this induction process. Organ specificity was also observed when partial induction at different dox concentrations was examined, as exemplified for three organs in Fig. 3B. These data strongly suggest that tissue/organ-specific calibrations are necessary to define conditions proper for partial activation of a gene in a given tissue. With the rTA system, partial induction between 6% and 53% was observed in various organs when the dox concentration in the drinking water was reduced from 2 mg/ml to 200  $\mu$ g/ml (21).

The full potential of the tet regulatory systems becomes evident in experiments where the synthesis of tTA was directed to the liver by P<sub>LAP</sub>. A particularly high tissue specificity of tTA synthesis is achieved in the LT2 mouse line. When crossed with L7 animals whose luciferase background in the liver is lowest, activation of luciferase exceeding five orders of magnitude is measured in that organ, two to four orders of magnitude higher than in any other organ.

*In situ* hybridization reveals an even distribution of mRNA of tTA as well as of luciferase throughout the organ and only the luciferase RNA synthesis is sensitive to the presence of dox. The specificity of P<sub>LAP</sub>-controlled tTA is revealed by analyzing offsprings of crosses between TA<sup>LAP1</sup> mice and animals carrying the  $\beta$ -galactosidase gene under the control of P<sub>hCMV</sub>-1. In such mice, hepatocytes are homogeneously stained (Fig.

5C), whereas cells of vessels and connective tissue show no  $\beta$ -galactosidase activity (21). In presence of dox, an equally homogeneous shut off of  $\beta$ -galactosidase synthesis is seen. There is no sign of "mosaic expression" as discussed in other reports (10, 26). Since in the latter studies viral promoters were used to drive tTA synthesis, the observed phenomenon is most likely due to specific properties of such promoters as described (27).

The main conclusion from our findings is that the tet regulatory systems indeed permit the tight and tissue-specific control of individual genes in higher organisms, provided certain prerequisites are met. Given proper rtTA/tTA-responsive promoters, tightness of an expression unit depends on the site of chromosomal integration. The further characterization of such chromosomal sites for gene targeting will, therefore, be of interest. Similarly, since the tissue-specific synthesis of rtTA or tTA depends on the proper combinations of promoter and chromosomal integration site, such combinations have to be identified. For hepatocyte-specific regulation, the combination of  $P_{LAP}$  with the locus of the  $TA^{LAP1}$  line already appears to be an excellent solution. Although it may be difficult at present to find such combinations for a particular question, mouse lines where Tc-controlled transactivators are synthesized tissue-specifically in the liver ( $TA^{LAP2}$ ), in heart muscle (11), or in pancreatic  $\beta$  cells (12) do already exist and lines with other specificities will become increasingly available. Moreover, chromosomal loci like the one revealed in the L7 line may upon further characterization become accessible for gene targeting. Such possibilities should facilitate the generation of defined animal models suitable for high-resolution studies of gene function *in vivo*.

We thank Dr. U. Schibler for the LAP promoter and for stimulating discussions. We are grateful to Dr. A. Bürki for help in preparing the first indicator mouse lines, to Dr. S. Bachman for the histologic examination of Tc-treated mice, and to Mrs. U. Amtmann for preparing the cryosections. We gratefully acknowledge Dr. Sabine Freundlieb's and Dr. Peter Seeburg's constructive comments as well as Mrs. Sibylle Reinig's patient help in preparing the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 229) and by the Fonds der Chemischen Industrie Deutschlands.

1. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
2. Sternberg, N. & Hamilton, D. (1981) *J. Mol. Biol.* **150**, 467–486.
3. Byrne, G. & Ruddle, F. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5473–5477.
4. Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E. J., Manning, R. W., Yu, S. H., Mulder, K. L. & Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6232–6236.
5. Gu, H., Zou, Y. R. & Rajewsky, K. (1993) *Cell* **73**, 1155–1164.
6. Gu, H., Marth, I. D., Orban, P. C., Mossann, H. & Rajewsky, K. (1994) *Science* **265**, 103–106.
7. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551.
8. Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W. & Bujard, H. (1995) *Science* **268**, 1766–1769.
9. Weinmann, P., Gossen, M., Hillen, W., Bujard, H. & Gatz, C. (1994) *Plant J.* **5**, 559–569.
10. Furth, P. A., Onge, L. St., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. & Hennighausen, L. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9302–9306.
11. Passman, R. S. & Fishman, G. I. (1994) *J. Clin. Invest.* **94**, 2421–2425.
12. Efrat, S., Fusco-DeMane, D., Lemberg, H., Emran, O. A. & Wang, X. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 3576–3580.
13. Talbot, D., Descombes, P. & Schibler, U. (1994) *Nucleic Acids Res.* **22**, 756–766.
14. Hogan, B., Constantini, F. & Lacy, E. (1995) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
17. Shockett, P., Difilippantonio, M., Hellman, N. & Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6522–6526.
18. Arias, I. M., Jakoby, W. B., Papper, H., Schachter, D. & Shafritz, D. A. (1987) *The Liver: Biology and Pathobiology* (Raven, New York), 2nd Ed.
19. Wisden, W., Morris, B. J. & Hunt, S. P. (1991) in *Molecular Neurobiology: A Practical Approach*, eds. Chad, J. & Wheal, H. (Oxford Univ. Press, New York), Vol. 2, pp. 205–225.
20. Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B. & Schaffner, W. (1985) *Cell* **41**, 521–530.
21. Kistner, A. (1996) Ph.D. thesis (Universität Heidelberg, Heidelberg, Germany).
22. Furth, P. A., Hennighausen, K., Baker, C., Beatty, B. & Woychick, R. (1991) *Nucleic Acids Res.* **19**, 6205–6208.
23. Yin, D. X., Zhu, L. & Schimke, R. T. (1996) *Anal. Biochem.* **235**, 195–201.
24. Dhawan, J., Rando, T. A., Elson, S. L., Bujard, H. & Blau, H. (1995) *Somatic Cell Mol. Genet.* **21**, 233–240.
25. Chrast-Balz, J. & Hooft van Huijsduijnen, R. (1996) *Nucleic Acids Res.* **24**, 2900–2904.
26. Hennighausen, L., Wall, R. J., Tillmann, W., Li, M. & Furth, P. (1995) *J. Cell. Biochem.* **59**, 1–10.
27. Baskar, J. F., Smith, P. P., Gajahan, N., Ray, A. J., Hoffman, S., Peffer, N., Tenney, D. J., Coleberg-Poley, A. M., Ghazal, P. & Nelson, J. A. (1996) *J. Virol.* **70**, 3207–3214.